

# Mechanism of activation of protein kinase B by insulin and IGF-1

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**Insulin activated endogenous protein kinase B $\alpha$  (also known as RAC/Akt kinase) activity 12-fold in L6 myotubes, while after transfection into 293 cells PKB $\alpha$  was activated 20- and 50-fold in response to insulin and IGF-1 respectively. In both cells, the activation of PKB $\alpha$  was accompanied by its phosphorylation at Thr308 and Ser473 and, like activation, phosphorylation of both of these residues was prevented by the phosphatidylinositol 3-kinase inhibitor wortmannin. Thr308 and/or Ser473 were mutated to Ala or Asp and activities of mutant PKB $\alpha$  molecules were analysed after transfection into 293 cells. The activity of wild-type and mutant PKB $\alpha$  was also measured *in vitro* after stoichiometric phosphorylation of Ser473 by MAPKAP kinase-2. These experiments demonstrated that activation of PKB $\alpha$  by insulin or insulin-like growth factor-1 (IGF-1) results from phosphorylation of both Thr308 and Ser473, that phosphorylation of both residues is critical to generate a high level of PKB $\alpha$  activity and that the phosphorylation of Thr308 *in vivo* is not dependent on phosphorylation of Ser473 or vice versa. We propose a model whereby PKB $\alpha$  becomes phosphorylated and activated in insulin/IGF-1-stimulated cells by an upstream kinase(s).**

**Keywords:** insulin signalling/phosphatidylinositol 3-kinase/protein kinase B/protein phosphorylation

## Introduction

Protein kinase B (PKB $\alpha$ ) (Coffer and Woodgett, 1991), also known as RAC $\alpha$  (Jones *et al.*, 1991a) and c-Akt (Bellacosa *et al.*, 1991), is the cellular homologue of v-Akt, a protein encoded in the genome of the AKT-8 acute transforming retrovirus isolated from a rodent T cell lymphoma (Staal *et al.*, 1977). Two other isoforms of PKB, termed PKB $\beta$  (Jones *et al.*, 1991b; Cheng *et al.*, 1992) and PKB $\gamma$  (Konishi *et al.*, 1995), have been identified. PKB $\beta$ , also known as RAC $\beta$  and Akt-2, is overexpressed in a significant number of ovarian (Cheng *et al.*, 1992) and pancreatic (Cheng *et al.*, 1996) cancers and PKB $\alpha$  is overexpressed in the breast cancer epithelial cell line MCF7 (Jones *et al.*, 1991a). PKB is composed of an N-terminal pleckstrin homology (PH) domain, followed

by a catalytic domain and a short C-terminal tail. The catalytic domain is most similar to cyclic AMP-dependent protein kinase (PKA; 65% similarity) and to protein kinase C (PKC; 75% similarity), findings that gave rise to two of its names, namely PKB (i.e. between PKA and PKC) and RAC (related to A and C kinase).

Many growth factors trigger the activation of phosphatidylinositol (PI) 3-kinase, the enzyme which converts PI 4,5-bisphosphate (PIP<sub>2</sub>) to the putative second messenger PI 3,4,5-trisphosphate (PIP<sub>3</sub>) and much of the current interest in PKB stems from the report that this enzyme lies downstream of PI 3-kinase (Franke *et al.*, 1995). PKB $\alpha$  is converted from an inactive to an active form with a half time of ~1 min when cells are stimulated with PDGF (Franke *et al.*, 1995), EGF or basic FGF (Burgering and Coffer, 1995) or insulin (Cross *et al.*, 1995; Kohn *et al.*, 1995) or pervanadate (Andjelkovic *et al.*, 1996). Activation of PKB by insulin or growth factors is prevented if the cells are preincubated with inhibitors of PI 3-kinase (wortmannin or LY 294002) or by overexpression of a dominant negative mutant of PI 3-kinase (Burgering and Coffer, 1995). Mutation of the tyrosine residues in the PDGF receptor that (when phosphorylated) bind to PI 3-kinase also prevent the activation of PKB $\alpha$  (Burgering and Coffer, 1995; Franke *et al.*, 1995). Furthermore, expression of a constitutively activated form of PI 3-kinase in cells has been shown to trigger the activation of PKB $\alpha$  (Didchenko *et al.*, 1996; Klippel *et al.*, 1996).

The protein kinase GSK3 has been identified as a likely physiological target for PKB $\alpha$ . GSK3 is inhibited in response to insulin with a half time of 2 min, slightly slower than the half time for activation of PKB $\alpha$  (1 min). Inhibition of GSK3 by insulin results in its phosphorylation at the same serine residue which is targeted by PKB $\alpha$  *in vitro*. Like the activation of PKB $\alpha$ , the inhibition of GSK3 by insulin is prevented by wortmannin and LY 294002. Inhibition of GSK3 is thought to contribute to the stimulation of glycogen synthesis (Cross *et al.*, 1995) and translation of certain mRNAs by insulin (Welsh *et al.*, 1994).

In the presence of phosphatidylserine, PKB $\alpha$  binds to PIP<sub>3</sub> with submicromolar affinity (James *et al.*, 1996; M.Frech, M.Andjelkovic, J.R.Falek and B.A.Hemmings, manuscript submitted). PI 4,5-bisphosphate and PI 3,4-bisphosphate bind to PKB $\alpha$  with lower affinities and PI 3,5-bisphosphate and PI 3-phosphate did not bind at all under these conditions (James *et al.*, 1996). The region of PKB $\alpha$  that interacts with PIP<sub>3</sub> is almost certainly the PH domain, because the isolated PH domain of PKB $\alpha$  binds PIP<sub>3</sub> with similar affinity to PKB $\alpha$  itself (M.Frech, M.Andjelkovic, J.R.Falek and B.A.Hemmings, manuscript submitted) and because the PH domain of several other proteins, such as the PH domains of  $\beta$ -spectrin and phospholipase C $\delta$ 1, are known to interact specifically with

other phosphoinositides (Hyvonen *et al.*, 1995; Lemmon *et al.*, 1995).

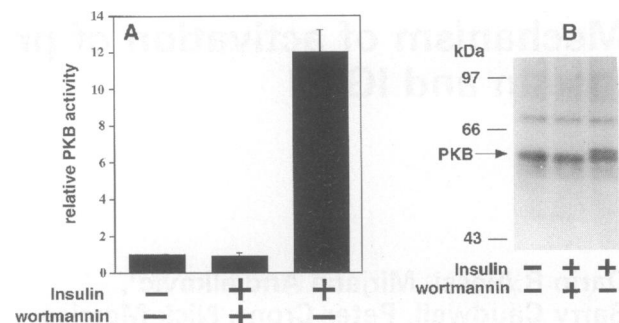
Although PIP<sub>3</sub> interacts strongly and relatively specifically with PKB $\alpha$  *in vitro* and the presence of the PH domain is necessary for significant activation of PKB $\alpha$  *in vivo* (Franke *et al.*, 1995; Andjelkovic *et al.*, 1996), no activation of PKB $\alpha$  by PIP<sub>3</sub> or other inositol phospholipids was detected in one study (James *et al.*, 1996), whereas in another (Frech *et al.*, 1996) PIP<sub>3</sub> was found to inhibit PKB $\alpha$  activity *in vitro*. An earlier report suggested that PI 3-phosphate activates PKB $\alpha$  directly *in vitro* (Franke *et al.*, 1995), but we were unable to confirm this finding (James *et al.*, 1996). Thus, although the interaction of PIP<sub>3</sub> with PKB $\alpha$  may initiate the activation process, perhaps by recruiting PKB $\alpha$  to the plasma membrane, it appears to be insufficient for activation, suggesting the involvement of another mechanism to activate PKB $\alpha$ . Cell stimulation with insulin, growth factors or pervanadate induces the hyperphosphorylation of PKB $\alpha$ , and treatment of this kinase with the serine/threonine-specific protein phosphatase PP2A, but not tyrosine-specific protein phosphatases, causes its inactivation (Andjelkovic *et al.*, 1996), indicating that serine/threonine phosphorylation is essential for PKB $\alpha$  activity. In this paper we report that PKB $\alpha$  becomes phosphorylated at Thr308 and Ser473 in response to insulin or insulin-like growth factor-1 (IGF-1), that the phosphorylation of both these residues is required to generate a high level of PKB $\alpha$  activity and that phosphorylation of both Thr308 and Ser473 is prevented by inhibitors of PI 3-kinase. The mechanism of action of PKB $\alpha$  is discussed in the light of these results.

## Results

### Activation of endogenous PKB by insulin in L6 myotubes is accompanied by phosphorylation of Thr308 and Ser473

<sup>32</sup>P-Labelled L6 myotubes were treated with or without 100 nM wortmannin for 10 min, then stimulated for 5 min with insulin or buffer. Under these conditions, insulin stimulation resulted in a 12-fold activation of endogenous PKB $\alpha$  (Figure 1A) and was accompanied by a  $1.9 \pm 0.3$ -fold increase in <sup>32</sup>P-labelling (four experiments) and retardation of its mobility on SDS-polyacrylamide gels (Figure 1B). The activation of PKB $\alpha$ , the increase in its <sup>32</sup>P-labelling and reduction in electrophoretic migration were all abolished by prior incubation of the cells with 100 nM wortmannin. Phosphoamino acid analysis of the whole protein revealed that <sup>32</sup>P-labelled PKB $\alpha$  was phosphorylated at both serine and threonine residues and that stimulation with insulin increased the <sup>32</sup>P-labelling of both phosphoamino acids (data not shown), in agreement with the results of Andjelkovic *et al.* (1996).

<sup>32</sup>P-Labelled PKB $\alpha$  was treated with 4-vinylpyridine to alkylate cysteine residues, digested with trypsin and the resulting <sup>32</sup>P-labelled peptides separated by chromatography on a C18 column. No major <sup>32</sup>P-labelled peptides were recovered from <sup>32</sup>P-labelled PKB $\alpha$  derived from unstimulated L6 myotubes (Figure 2A), indicating that, in the absence of insulin, there was a low level phosphorylation at a number of sites. However, following stimulation with insulin, two major <sup>32</sup>P-labelled peptides were observed, termed A and B (Figure 2B), whose <sup>32</sup>P-labelling

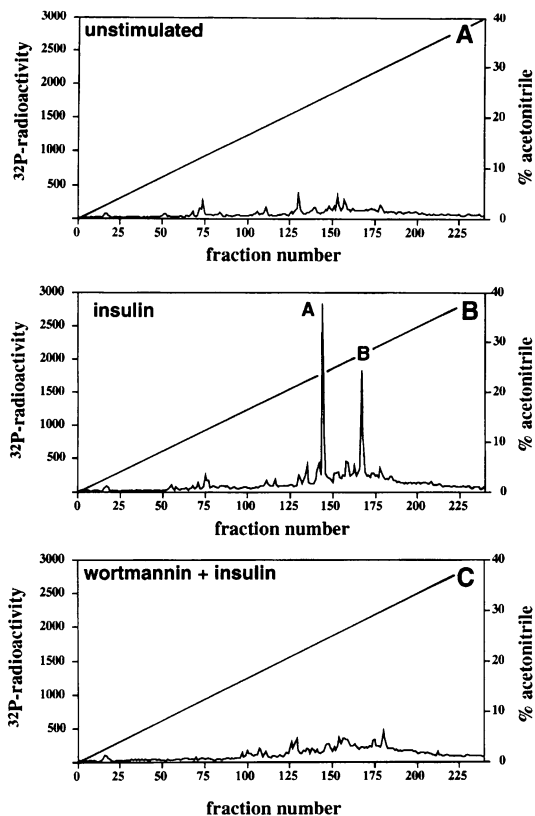


**Fig. 1.** Insulin induces activation and phosphorylation of PKB $\alpha$  in L6 myotubes. Three 10 cm dishes of L6 myotubes were <sup>32</sup>P-labelled and treated for 10 min with or without 100 nM wortmannin and then for 5 min with or without 100 nM insulin as described under Materials and methods. (A) PKB $\alpha$  was immunoprecipitated from the lysates and an aliquot (15%) assayed for PKB $\alpha$  activity. The activities are plotted  $\pm$  SEM for three experiments relative to PKB $\alpha$  derived from unstimulated cells, which was 10 mU/mg. (B) The remaining 85% of the immunoprecipitated PKB $\alpha$  was alkylated with 4-vinylpyridine, electrophoresed on a 10% polyacrylamide gel (prepared without SDS to enhance the phosphorylation-induced decrease in mobility) and autoradiographed. The positions of the molecular mass markers glycogen phosphorylase (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa) are marked.

was prevented if the myotubes were first preincubated with wortmannin (Figure 2C). Peptide A was phosphorylated predominantly on serine while peptide B was labelled on threonine (Figure 3A). Phosphopeptide A was further purified by chromatography on a microbore C18 column at pH 6.5 as described in the legend to Figure 3, and amino acid sequencing established that it commenced at residue 465. Only a single burst of <sup>32</sup>P radioactivity was observed after the eighth cycle of Edman degradation (Figure 3B), demonstrating that insulin stimulation of L6 myotubes had triggered phosphorylation of PKB $\alpha$  at Ser473, which is located nine residues from the C-terminus of the protein. Phosphopeptide B was only recovered in significant amounts if <sup>32</sup>P-labelled PKB $\alpha$  was treated with 4-vinylpyridine prior to digestion with trypsin, indicating that this peptide contained a cysteine residue(s), and a single burst of <sup>32</sup>P radioactivity was observed after the first cycle of Edman degradation (Figure 3C). This suggested that the site of phosphorylation was residue 308, since it is the only threonine in PKB $\alpha$  that follows a lysine or arginine residue and which is located in a tryptic peptide containing a cysteine residue (at position 310). The acetonitrile concentration at which phosphopeptide B is eluted from the C18 column (28%) and its isoelectric point (4.0) are also consistent with its assignment as the peptide comprising residues 308–325 of PKB $\alpha$ . The poor recoveries of peptide B during further purification at pH 6.5 prevented determination of its amino acid sequence, but further experiments described below using transiently transfected 293 cells established that this peptide does correspond to residues 308–325 of PKB $\alpha$ .

### Mapping the phosphorylation sites of PKB $\alpha$ in transiently transfected 293 cells

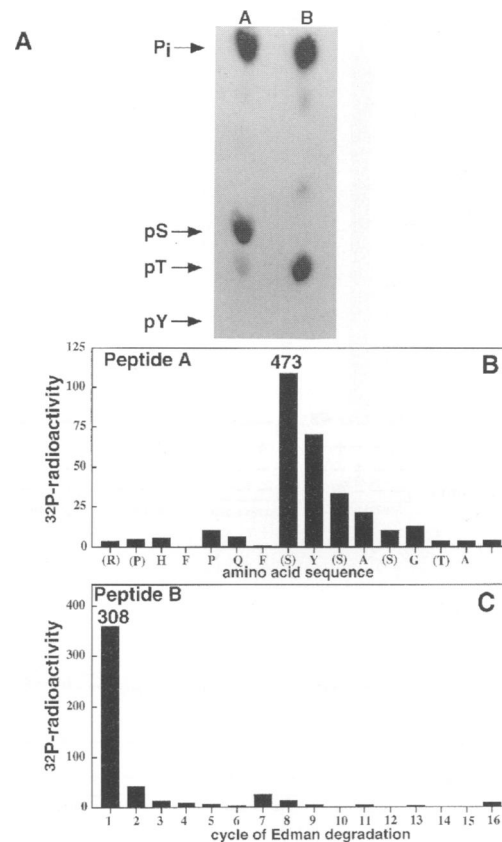
In order to establish that Thr308 was a major site phosphorylated on PKB $\alpha$  in response to insulin, it was necessary to transfect PKB $\alpha$  into an insulin-sensitive cell, in order to obtain higher levels of the enzyme. Haemagglutinin epitope-tagged PKB $\alpha$  (HA-PKB $\alpha$ ) was therefore



**Fig. 2.** Insulin stimulation of L6 myotubes induces phosphorylation of two peptides in PKB $\alpha$ . Bands corresponding to  $^{32}\text{P}$ -labelled PKB $\alpha$  from Figure 1B were excised from the gel, digested with trypsin and chromatographed on a Vydac 218TP54 C18 column (Separations Group, Hesperia, CA) equilibrated with 0.1% v/v trifluoroacetic acid (TFA) and the columns developed with a linear acetonitrile gradient (diagonal line). The flow rate was 0.8 ml/min and fractions of 0.4 ml were collected. (A) Tryptic peptide map of  $^{32}\text{P}$ -labelled PKB $\alpha$  from unstimulated L6 myotubes. (B) Tryptic peptide map of  $^{32}\text{P}$ -labelled PKB $\alpha$  from insulin-stimulated L6 myotubes. (C) Tryptic peptide map of  $^{32}\text{P}$ -labelled PKB $\alpha$  from L6 myotubes treated with wortmannin prior to insulin. The two major  $^{32}\text{P}$ -labelled peptides eluting at 23.7 and 28% acetonitrile are named peptides A and B respectively. Similar results were obtained in four (A and B) and two (C) experiments.

transfected into human embryonic kidney 293 cells and subsequent stimulation with insulin and IGF-1 resulted in 20- and 46-fold activation of transfected PKB $\alpha$  respectively (Figure 4A). The half time for activation was 1 min, as found with other cells. Activation of PKB $\alpha$  by insulin or IGF-1 was prevented by prior incubation with wortmannin (Figure 4A) and no activation occurred if 293 cells were transfected with vector alone and then stimulated with insulin or IGF-1 (data not shown).

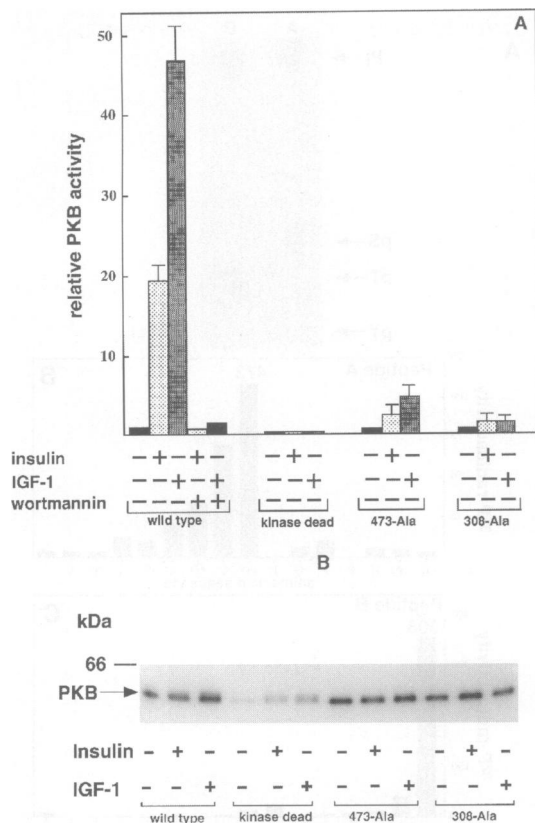
293 cells transiently expressing HA-PKB $\alpha$  were  $^{32}\text{P}$ -labelled and stimulated with buffer or IGF-1. The  $^{32}\text{P}$ -labelled HA-PKB $\alpha$  was immunoprecipitated, digested with trypsin and the resulting peptides analysed by C18 chromatography. Two prominent  $^{32}\text{P}$ -labelled peptides were present in unstimulated 293 cells (Figure 5A). One, termed peptide C, usually eluted as a doublet at 20–21% acetonitrile, while the other, termed peptide F, eluted at 29.7% acetonitrile. Stimulation with insulin or IGF-1 did not affect the  $^{32}\text{P}$ -labelling of peptides C and F (Figure 5A and B), but induced  $^{32}\text{P}$ -labelling of two new peptides, termed D (23.4% acetonitrile) and E (28% acetonitrile), which eluted at the same acetonitrile concentrations as



**Fig. 3.** Identification of the phosphorylation sites in peptides A and B. (A) Peptides A and B from Figure 2B (1000 c.p.m.) were incubated for 90 min at 110°C in 6 M HCl, electrophoresed on thin layer cellulose at pH 3.5 to resolve orthophosphate (Pi), phosphoserine (pS), phosphothreonine (pT) and phosphotyrosine (pY) and autoradiographed. (B) Peptide A (Figure 2B) obtained from 50 10-cm dishes of  $^{32}\text{P}$ -labelled L6 myotubes was further purified by chromatography on a microbore C18 column equilibrated in 10 mM ammonium acetate, pH 6.5, instead of 0.1% TFA. A single peak of  $^{32}\text{P}$  radioactivity was observed at 21% acetonitrile, which coincided with a peak of 214 nm absorbance. Of the sample, 80% (1 pmol) was analysed on an Applied Biosystems 476A sequencer to determine the amino acid sequence and the phenylthiohydantoin (Pth) amino acids identified after each cycle of Edman degradation are shown using the single letter code. The residues in parentheses were not present in sufficient amounts to be identified unambiguously. In order to identify the site(s) of phosphorylation, the remaining 20% of the sample (600 c.p.m.) was then coupled covalently to a Sequelon arylamine membrane and analysed on an Applied Biosystems 470A sequencer using the modified programme described by Stokoe *et al.* (1992).  $^{32}\text{P}$  radioactivity was measured after each cycle of Edman degradation. (C) Peptide B from Figure 2B (800 c.p.m.) was subjected to solid phase sequencing as in (B).

peptides A and B derived from the endogenous PKB $\alpha$  from L6 myotubes (Figure 2B) and had the same isoelectric points (7.2 and 4.0 respectively). Treatment of 293 cells expressing HA-PKB $\alpha$  with 100 nM wortmannin prior to stimulation with IGF-1 prevented phosphorylation of peptides D and E, but had no effect on the  $^{32}\text{P}$ -labelling of peptides C and F (Figure 5C).

Peptides C–F were further purified by rechromatography on the C18 column at pH 6.5 and sequenced. Peptide C gave rise to three separate (but closely eluting)  $^{32}\text{P}$ -labelled peptides (data not shown). Amino acid sequencing revealed that all three commenced at residue 122 of PKB $\alpha$  and that Ser124 was the site of phosphorylation (Figure 6A).



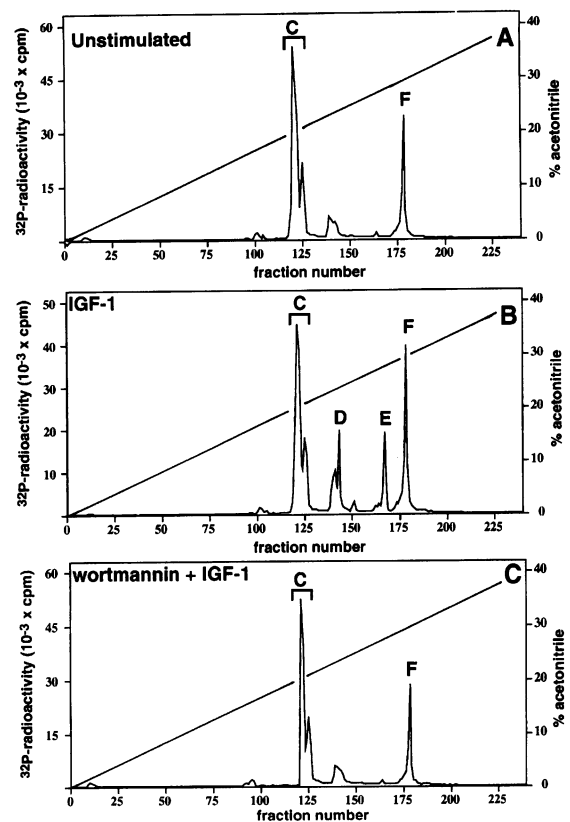
**Fig. 4.** Effect of mutation of PKB $\alpha$  on activation by insulin or IGF-1 in 293 cells. 293 cells were transiently transfected with DNA constructs expressing wild-type PKB $\alpha$ , HA-KD PKB $\alpha$ , HA-473A PKB $\alpha$  or HA-308A PKB $\alpha$ . After treatment for 10 min with or without 100 nM wortmannin, the cells were stimulated for 10 min with or without 100 nM insulin or 50 ng/ml IGF-1 in the continued presence or absence of wortmannin. PKB $\alpha$  was immunoprecipitated from the lysates and assayed and activities corrected for the relative levels of expression of each HA-PKB $\alpha$ . The results are expressed as fold activation relative to the specific activity of wild-type HA-PKB $\alpha$  from unstimulated 293 cells ( $25 \pm 5$  U/mg). (B) Aliquots of 20  $\mu$ g protein from each lysate were electrophoresed on a 10% SDS-polyacrylamide gel and immunoblotted using monoclonal HA antibody. The molecular markers are those used in Figure 1B.

Peptide D only contained phosphoserine and, as expected, corresponded to the PKB $\alpha$  tryptic peptide commencing at residue 465 that was phosphorylated at Ser473 (Figure 6B). Peptide E contained only phosphothreonine and amino acid sequencing demonstrated that it corresponded to residues 308–325, the phosphorylation site being Thr308 (Figure 6C). Peptide F contained only phosphothreonine and corresponded to the peptide commencing at residue 437 of PKB $\alpha$  phosphorylated at Thr450 (Figure 6D).

The experiments described above were repeated using insulin instead of IGF-1. The results were identical, except that the  $^{32}$ P-labelling of peptides D and E was ~50% of the levels observed with IGF-1 (data not shown). This is consistent with the 2-fold lower level of activation of PKB $\alpha$  by insulin compared with IGF-1 (Figure 3A).

#### MAPKAP kinase-2 phosphorylates Ser473 of PKB $\alpha$ causing partial activation

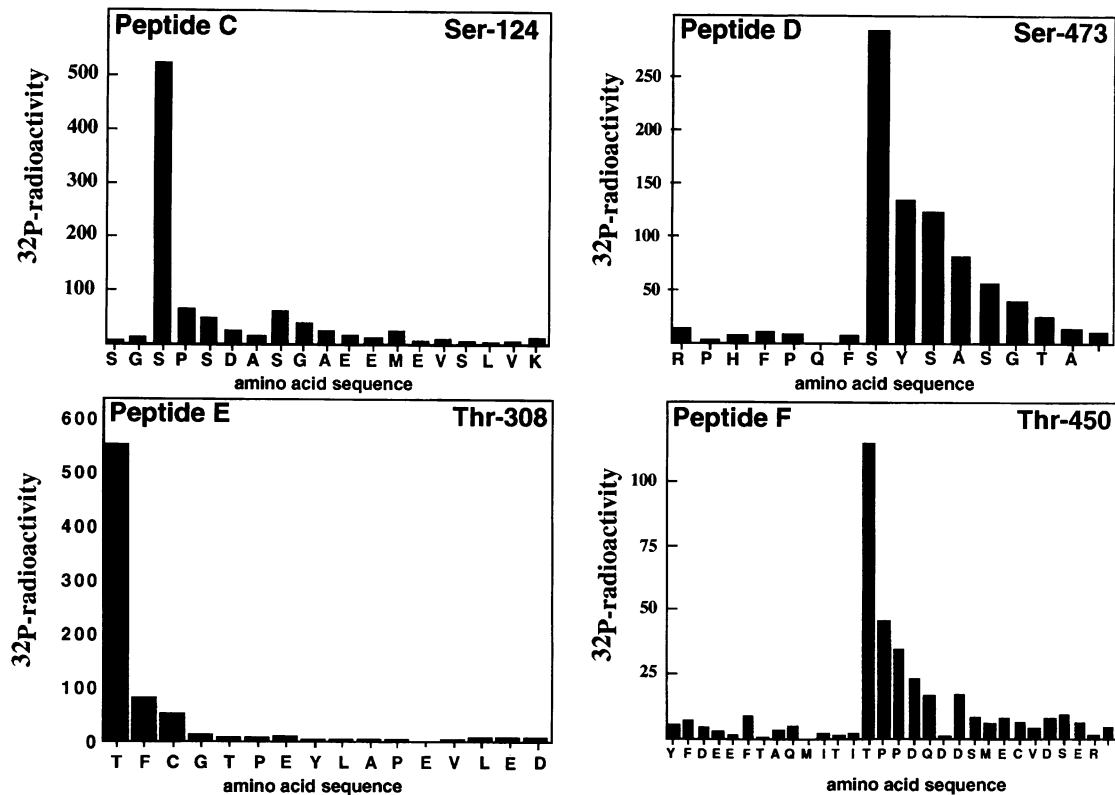
Ser473 of PKB $\alpha$  lies in a consensus sequence Phe-X-X-Phe/Tyr-Ser/Thr-Phe/Tyr which is conserved in several protein kinases that participate in signal transduction



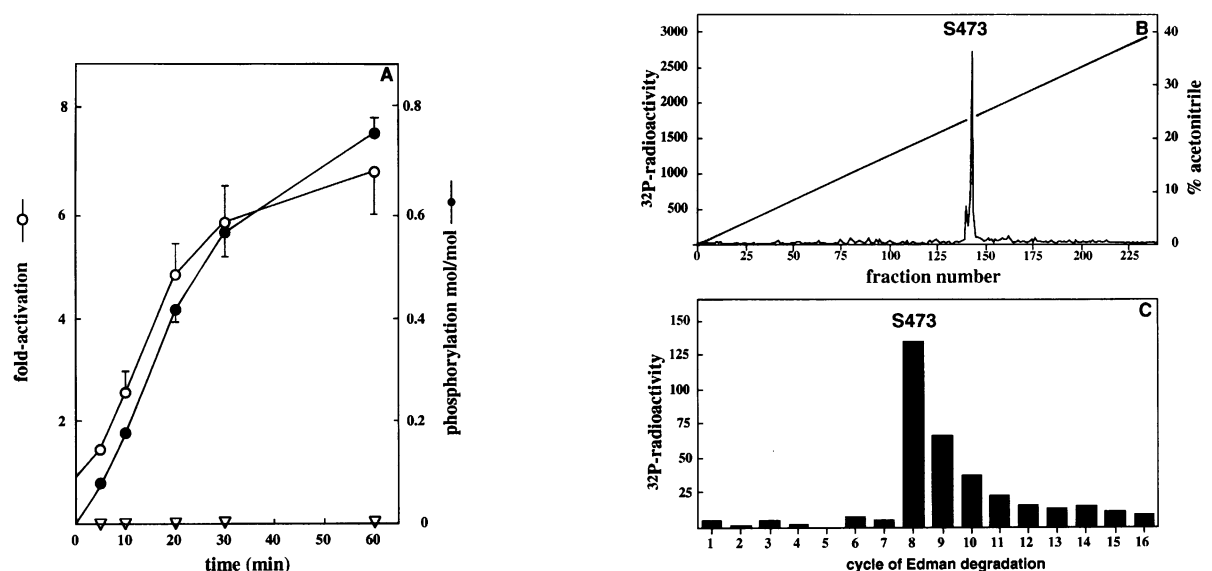
**Fig. 5.** IGF-1 stimulation of 293 cells induces the phosphorylation of two peptides in transfected HA-PKB $\alpha$ . 293 cells transiently transfected with wild-type HA-PKB $\alpha$  DNA constructs were  $^{32}$ P-labelled, treated for 10 min without (A and B) or with (C) 100 nM wortmannin and then for 10 min without (A) or with (B and C) 50 ng/ml IGF-1. The  $^{32}$ P-labelled HA-PKB $\alpha$  was immunoprecipitated from the lysates, treated with 4-vinylpyridine, electrophoresed on a 10% polyacrylamide gel, excised from the gel and digested with trypsin (see Materials and methods). Subsequent chromatography on a C18 column as described in the legend to Figure 2 resolved four major phosphopeptides termed C–F. Similar results were obtained in six separate experiments for (A) and (B) and in two experiments for (C).

pathways (Pearson *et al.*, 1995). We therefore synthesized a peptide corresponding to residues 465–478 of PKB $\alpha$  and used it to purify to near homogeneity the major Ser473 kinase from rabbit skeletal muscle extracts. This enzyme was identified as MAP kinase-activated protein (MAPKAP) kinase-2 (Stokoe *et al.*, 1992), a component of a stress- and cytokine-activated MAP kinase cascade (Rouse *et al.*, 1994; Cuenda *et al.*, 1995). Although MAPKAP kinase-2 does not appear to be a physiologically relevant Ser473 kinase (see Discussion), this finding was useful because it allowed us to establish the effect of Ser473 phosphorylation on PKB $\alpha$  activity *in vitro*.

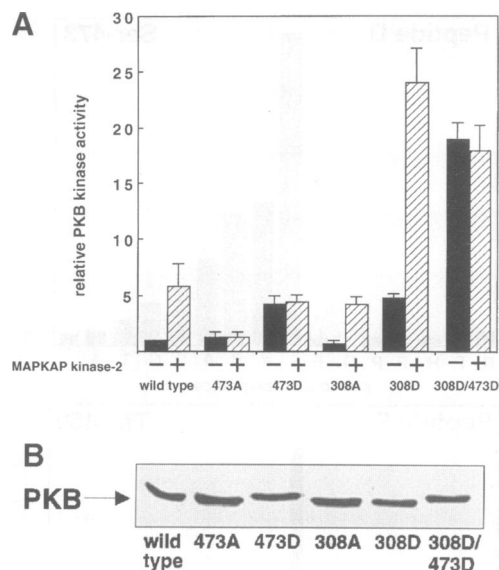
MAPKAP kinase-2 phosphorylated wild-type HA-PKB $\alpha$  (Figure 7A) or catalytically inactive HA-PKB $\alpha$  in which Lys179 had been mutated to Ala (data not shown) to a level approaching 1 mol/mol protein. Phosphorylation of wild-type PKB $\alpha$  was paralleled by a 7-fold increase in activity, whereas phosphorylation of the catalytically inactive mutant did not cause any activation (Figure 7A). No phosphorylation or activation of wild-type HA-PKB $\alpha$  occurred if MAPKAP kinase-2 or MgATP was omitted from the reaction (data not shown). Wild-type HA-PKB $\alpha$



**Fig. 6.** Identification of the phosphorylated residues in peptides C–F. The  $^{32}\text{P}$ -labelled tryptic peptides from Figure 5B were further purified by C18 chromatography at pH 6.5 as described in the legend to Figure 3 (peptides C, D and F) or by chromatography on a microbore C18 column equilibrated in 0.1% TFA, pH 1.9 (peptide E). Peptide sequencing and identification of phosphorylation sites was also carried out as in Figure 3.



**Fig. 7.** MAPKAP kinase-2 phosphorylates Ser473 specifically and partially activates PKB $\alpha$ . HA-PKB $\alpha$  was immunoprecipitated from the lysates of unstimulated COS-1 cells. (A) Aliquots of 0.5  $\mu\text{g}$  immunoprecipitated HA-PKB $\alpha$  were incubated with MAPKAP kinase-2 (50 U/ml), 10 mM magnesium acetate and 100 mM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  in a total of 40  $\mu\text{l}$  buffer B. At various times, aliquots were removed and either assayed for PKB $\alpha$  activity (open circles) or for incorporation of phosphate into PKB $\alpha$  (closed circles). Before measuring PKB $\alpha$  activity, EDTA was added to a final concentration of 20 mM to stop the reaction and the immunoprecipitates washed twice with 1 ml buffer B containing 0.5 M NaCl, then twice with 1 ml buffer B to remove MAPKAP kinase-2. The results are presented as  $\pm$  SEM for six determinations (two separate experiments) and PKB $\alpha$  activities are presented relative to control experiments in which HA-PKB $\alpha$  was incubated with MgATP in the absence of MAPKAP kinase-2 (which caused no activation). Phosphorylation was assessed by counting the  $^{32}\text{P}$  radioactivity associated with the band of PKB $\alpha$  after SDS-PAGE. The open triangles show the activity of immunoprecipitated HA-KD PKB $\alpha$  phosphorylated by MAPKAP kinase-2. (B) HA-PKB $\alpha$  phosphorylated for 1 h with MAPKAP kinase-2 and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  as in (A) was digested with trypsin and chromatographed on a C18 column as described in the legend to Figure 2. (C) The major  $^{32}\text{P}$ -labelled peptide from (B) was analysed on the 470A sequencer as in Figure 3 to identify the site of phosphorylation.



**Fig. 8.** Activation of HA-PKB $\alpha$  mutants *in vitro* by MAPKAP kinase-2. (A) Wild-type and mutant HA-PKB $\alpha$  proteins were immunoprecipitated from the lysates of unstimulated COS-1 cells expressing these constructs and incubated for 60 min with MgATP in the absence (-, filled bars) or presence (+, hatched bars) of MAPKAP kinase-2 and MgATP (50 U/ml) essentially as described in Figure 7A. Specific activities were measured with Crosstide as the substrate (see Materials and methods). The data are presented as fold activation relative to wild-type HA-PKB $\alpha$  incubated in the absence of MAPKAP kinase-2 (30 mU/mg). The results are shown as the average  $\pm$  SEM for three experiments. (B) Aliquots of 20  $\mu$ g protein from each lysate were electrophoresed on a 10% SDS-polyacrylamide gel and immunoblotted using monoclonal HA-antibody.

that had been maximally activated with MAPKAP kinase-2 was completely dephosphorylated and inactivated by treatment with protein phosphatase 2A (data not shown).

HA-PKB $\alpha$  that had been maximally phosphorylated with MAPKAP kinase-2 was digested with trypsin and C18 chromatography revealed one major  $^{32}$ P-labelled phosphoserine-containing peptide (Figure 7B). This peptide eluted at the same acetonitrile concentration (Figure 7B) and had the same isoelectric point of 7.2 (data not shown) as the  $^{32}$ P-labelled tryptic peptide containing Ser473 (compare Figures 7B and 2B). Solid phase sequencing gave a burst of  $^{32}$ P radioactivity after the eighth cycle of Edman degradation (Figure 7C), establishing that Ser473 was the site of phosphorylation. The same [ $^{32}$ P]peptide was obtained after tryptic digestion of catalytically inactive HA-KD PKB $\alpha$  phosphorylated with MAPKAP kinase-2 (data not shown).

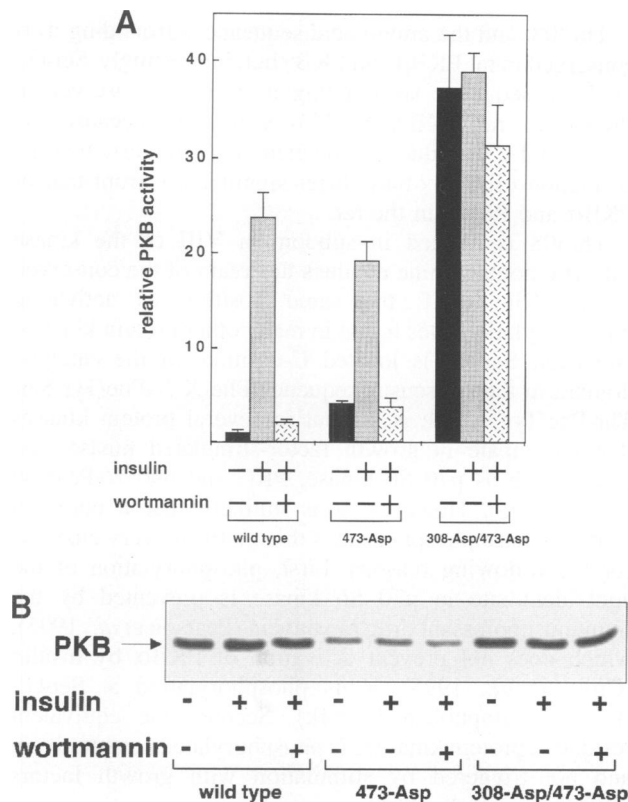
#### Phosphorylation of Thr308 and Ser473 causes synergistic activation of PKB $\alpha$

The experiments described above demonstrated that phosphorylation of Ser473 activates PKB $\alpha$  *in vitro*, but did not address the role of phosphorylation of Thr308 or how phosphorylation of Thr308 might influence the effect of Ser473 phosphorylation on activity, or vice versa. We therefore prepared HA-tagged PKB $\alpha$  DNA constructs in which either Ser473 or Thr308 were changed either to Ala (to block the effect of phosphorylation) or to Asp (to try and mimic the effect of phosphorylation). All the mutants were expressed at a similar level in serum-starved COS-1 cells (Figure 8B), transfection of this insulin-

insensitive cell line producing even higher levels of expression of PKB $\alpha$  than 293 cells. The effects of maximally phosphorylating wild-type and mutant PKB $\alpha$  at Ser473 is shown in Figure 8A. Before phosphorylation with MAPKAP kinase-2 the activity of HA-473A PKB $\alpha$  was similar to that of unstimulated wild-type HA-PKB $\alpha$  and, as expected, incubation with MAPKAP kinase-2 and MgATP did not result in any further activation of HA-473A PKB $\alpha$ . In contrast, the activity of HA-473D PKB $\alpha$  was 5- to 6-fold higher than that of unstimulated wild-type HA-PKB $\alpha$  protein and similar to that of wild-type HA-PKB $\alpha$  phosphorylated at Ser473. As expected, HA-473D PKB $\alpha$  was also not activated further by incubation with MAPKAP kinase-2 and MgATP (Figure 8A). The activity of HA-308A PKB $\alpha$  was ~40% of that of the unstimulated wild-type enzyme and after phosphorylation with MAPKAP kinase-2 its activity increased to a level similar to that of wild-type HA-PKB $\alpha$  phosphorylated at Ser473 (Figure 8A). Interestingly, HA-308D PKB $\alpha$ , which (like HA-473D PKB $\alpha$ ) was 5-fold more active than dephosphorylated wild-type HA-PKB $\alpha$ , was activated dramatically by phosphorylation of Ser473 (Figure 8A). After incubation with MAPKAP kinase-2 and MgATP, the activity of HA-308D PKB $\alpha$  was nearly 5-fold higher than that of wild-type HA-PKB $\alpha$  phosphorylated at Ser473 (Figure 8A). These results suggest that phosphorylation of either Thr308 or Ser473 leads to partial activation of PKB $\alpha$  *in vitro* and that phosphorylation of both residues results in a synergistic activation of the enzyme. This idea was supported by further experiments in which both Thr308 and Ser473 were changed to Asp. When this double mutant was expressed in COS-1 cells it was found to possess an 18-fold higher specific activity than the dephosphorylated wild-type protein. As expected, the activity of this mutant was not increased further by incubation with MAPKAP kinase-2 and MgATP (Figure 8A).

#### Phosphorylation of both Thr308 and Ser473 is required for a high level of activation of PKB $\alpha$ *in vivo*

HA-308A PKB $\alpha$  and HA-473A PKB $\alpha$  were transiently expressed into 293 cells. The basal level of activity of HA-473A PKB $\alpha$  derived from unstimulated cells was similar to that of wild-type PKB $\alpha$  (Figure 4A). Stimulation of 293 cells expressing HA-473A PKB $\alpha$  with insulin or IGF-1 increased the activity of this mutant 3- and 5-fold respectively; i.e. to 15% of the activity of wild-type HA-PKB $\alpha$  which had been transiently expressed and stimulated under identical conditions (Figure 4A). The basal activity of HA-308A PKB $\alpha$  in unstimulated cells was also similar to that of wild-type HA-PKB $\alpha$  derived from unstimulated cells, but virtually no activation of this mutant occurred following stimulation of the cells with insulin or IGF-1 (Figure 4A). These data are consistent with *in vitro* experiments (Figures 7A and 8) and indicate that maximal activation of PKB $\alpha$  requires phosphorylation of both Ser473 and Thr308 and that phosphorylation of both residues results in a synergistic activation of the enzyme. Consistent with these results, HA-473D PKB $\alpha$  displayed 5-fold higher activity and the HA-308D/HA-473D double mutant 40-fold higher activity than wild-type HA-PKB $\alpha$  when expressed in unstimulated cells (Figure 9). Following stimulation with insulin, HA-473D PKB $\alpha$  was activated

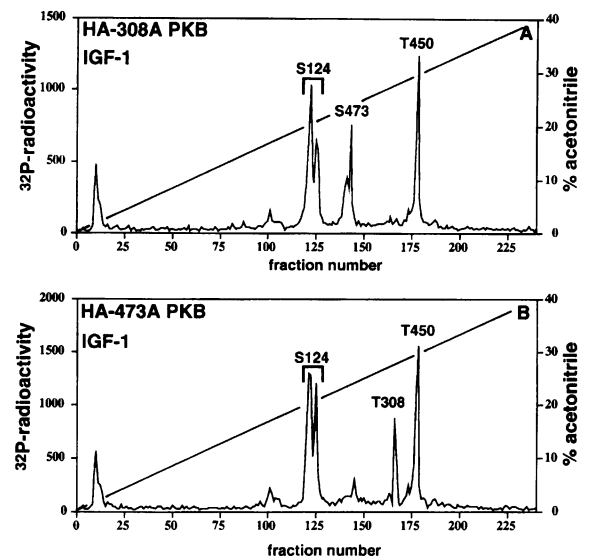


**Fig. 9.** Effect of mutation of PKB $\alpha$  on its activation by insulin in 293 cells. (A) 293 cells were transiently transfected with DNA constructs expressing wild-type PKB $\alpha$ , HA-473D PKB $\alpha$  and HA-308D/473D PKB $\alpha$ . After treatment for 10 min with or without 100 nM wortmannin, cells were stimulated for 10 min with or without 100 nM insulin in the continued presence or absence of wortmannin. PKB $\alpha$  was immunoprecipitated from the lysates and assayed with Crostide as substrate (see Materials and methods). The results are expressed as fold activation relative to the specific activity of wild-type HA-PKB $\alpha$  (0.03 U/mg) obtained from unstimulated 293 cells and the activities are corrected for the relative levels of HA-PKB $\alpha$  expression as described in Materials and methods. (B) Aliquots of 20  $\mu$ g protein from each lysate were electrophoresed on a 10% SDS-polyacrylamide gel and immunoblotted using monoclonal HA antibody.

to a level similar to that observed with the wild-type enzyme, while the HA-308D/HA-473D double mutant could not be activated further (Figure 9). As expected, activation of HA-473D PKB $\alpha$  by insulin was prevented by wortmannin and the activity of the HA-308D/HA-473D double mutant was resistant to wortmannin (Figure 9).

#### Phosphorylation of Thr308 is not dependent on phosphorylation of Ser473 or vice versa in 293 cells

$^{32}$ P-Labelled 293 cells were transfected with either HA-308A PKB $\alpha$  or HA-473A PKB $\alpha$ , then stimulated with either IGF-1 or buffer. The mutant PKB $\alpha$ s were immunoprecipitated from the extracts, digested with trypsin and chromatographed on a C18 column (Figure 10). These experiments demonstrated that IGF-1 stimulation induced phosphorylation of HA-473A PKB $\alpha$  at Thr308 and phosphorylation of HA-308A PKB $\alpha$  at Ser473. Similar results were obtained after stimulation with insulin rather than IGF-1 (data not shown).



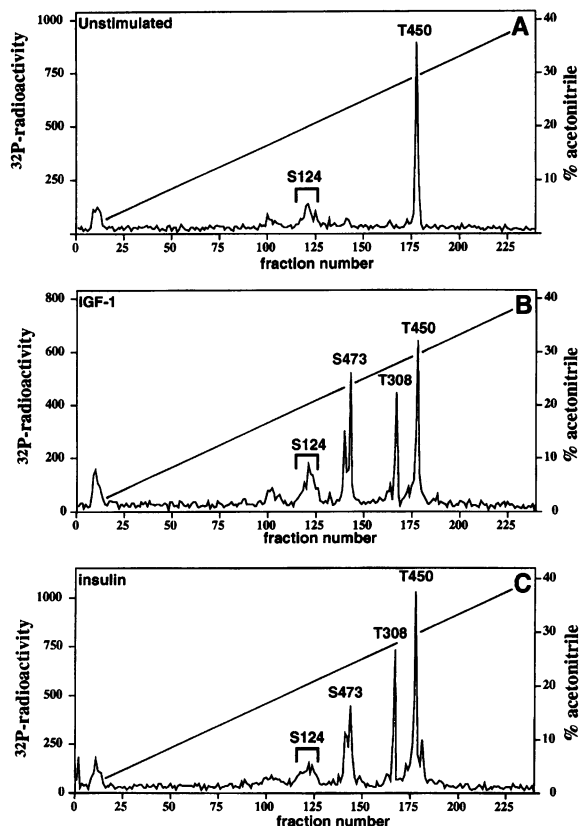
**Fig. 10.** Phosphorylation of Thr308 is not required for phosphorylation of Ser473 or vice versa. A 10 cm dish of 293 cells was transiently transfected with either HA-308A PKB $\alpha$  (A) or HA-473A PKB $\alpha$  (B) DNA constructs,  $^{32}$ P-labelled and stimulated for 10 min with 50 ng/ml IGF-1. The  $^{32}$ P-labelled PKB $\alpha$  mutants were immunoprecipitated from the lysates, treated with 4-vinylpyridine, electrophoresed on a 10% polyacrylamide gel, excised from the gel and digested with trypsin (see Materials and methods), then chromatographed on a C18 column as described in the legend to Figure 2. The tryptic peptides containing the phosphorylated residues Ser124, Thr308, Thr450 and Ser473 are marked and their assignments were confirmed by phosphoamino acid analysis and sequencing to identify the sites of phosphorylation (data not shown). The phosphopeptides containing Thr308 and Ser473 were absent if stimulation with IGF-1 was omitted, while the phosphopeptides containing Ser124 and Thr450 were present at similar levels as observed with wild-type PKB $\alpha$  (see Figure 5A). Similar results were obtained in three separate experiments.

#### IGF-1 or insulin induces phosphorylation of Thr308 and Ser473 in a catalytically inactive mutant of PKB $\alpha$

A 'kinase dead' mutant of PKB $\alpha$ , termed HA-KD-PKB $\alpha$ , in which Lys179 was changed to Ala (see above) was transiently expressed in 293 cells and its level of expression found to be several-fold lower than that of wild-type HA-PKB $\alpha$  expressed under identical conditions (Figure 4B). No PKB $\alpha$  activity was detected when 293 cells expressing HA-KD-PKB $\alpha$  were stimulated with insulin or IGF-1 (Figure 4A).

293 cells that had been transiently transfected with HA-KD-PKB $\alpha$  were  $^{32}$ P-labelled, then stimulated with buffer, insulin or IGF-1 and the sites on PKB $\alpha$  phosphorylated under these conditions were mapped. In contrast to wild-type HA-PKB $\alpha$  from unstimulated 293 cells (Figure 5), HA-KD PKB $\alpha$  was phosphorylated to a much lower level at Ser124, but phosphorylated similarly at Thr450 (Figure 11A). Following stimulation with IGF-1 (Figure 11B) or insulin (Figure 11C) HA-KD-PKB $\alpha$  became phosphorylated at the peptides containing Thr308 and Ser473, the extent of phosphorylation of these sites being at least as high as wild-type PKB $\alpha$ . Amino acid sequencing of these peptides established that they were phosphorylated at Thr308 and Ser473 respectively (data not shown). These results are considered further in the Discussion.





**Fig. 11.** The catalytically inactive PKB $\alpha$  mutant (HA-KD-PKB $\alpha$ ) expressed in 293 cells is phosphorylated at Thr308 and Ser473 after stimulation with IGF-1. Each 10 cm dish of 293 cells transiently transfected with HA-KD-PKB $\alpha$  DNA constructs was  $^{32}$ P-labelled and incubated for 10 min with buffer (A), 50 ng/ml IGF-1 (B) or 100 nM insulin (C). The  $^{32}$ P-labelled HA-KD-PKB $\alpha$  was immunoprecipitated from the lysates, treated with 4-vinylpyridine, electrophoresed on a 10% polyacrylamide gel, excised from the gel and digested with trypsin (see Materials and methods), then chromatographed on a C18 column as described in the legend to Figure 2. The tryptic peptides containing the phosphorylated residues Ser124, Thr308, Thr450 and Ser473 are marked. Similar results were obtained in three separate experiments for (A) and (B) and in two experiments for (C).

## Discussion

In this paper we have established that Thr308 and Ser473 are the major, if not the only, residues in PKB $\alpha$  that become phosphorylated in response to insulin or IGF-1 (Figures 2 and 5) and that phosphorylation of both residues is required to generate a high level of PKB $\alpha$  activity. Thus mutation of either Thr308 or Ser473 to Ala greatly decreased the activation of transfected PKB $\alpha$  by insulin or IGF-1 in 293 cells (Figure 4). Moreover, PKB $\alpha$  became partially active *in vitro* when either Thr308 or Ser473 were changed to Asp or when Ser473 was phosphorylated by MAPKAP kinase-2 *in vitro* and far more active when the D308 mutant of PKB $\alpha$  was phosphorylated by MAPKAP kinase-2 or when Thr308 and Ser473 were both mutated to Asp (Figures 8 and 9). Moreover, the D308/D473 double mutant could not be activated further by stimulating cells with insulin (Figure 9). These observations demonstrate that phosphorylation of Thr308 and Ser473 act synergistically to generate a high level of PKB $\alpha$  activity. The constitutively active D308/D473 double mutant should prove useful for future investigations of the physiological role(s) of PKB $\alpha$ .

Thr308, and the amino acid sequence surrounding it, is conserved in rat PKB $\beta$  and PKB $\gamma$  but, interestingly, Ser473 (and the sequence surrounding it) is only conserved in PKB $\beta$ . In rat PKB $\gamma$ , Ser473 is missing because the C-terminal 23 residues are deleted. This suggests that the regulation of PKB $\gamma$  may differ significantly from that of PKB $\alpha$  and PKB $\beta$  in the rat.

Thr308 is located in subdomain VIII of the kinase catalytic domain, nine residues upstream of the conserved Ala-Pro-Glu motif, the same position as activating phosphorylation sites found in many other protein kinases. However, Ser473 is located C-terminal of the catalytic domain in the consensus sequence Phe-X-X-Phe/Tyr-Ser/Thr-Phe/Tyr, which is present in several protein kinases that participate in growth factor-stimulated kinase cascades, such as p70 S6 kinase, PKC and p90<sup>rk</sup> (Pearson *et al.*, 1995). However, it is unlikely that a common protein kinase phosphorylates this motif in every enzyme, for the following reasons. First, phosphorylation of the equivalent site in p70 S6 kinase is prevented by the immunosuppressant drug rapamycin (Pearson *et al.*, 1995), which does not prevent activation of PKB $\alpha$  by insulin (Cross *et al.*, 1995) or its phosphorylation at Ser473 (D.Alessi, unpublished work). Second, the equivalent residue in protein kinase C is phosphorylated constitutively and not triggered by stimulation with growth factors (Tsutakawa *et al.*, 1995).

MAPKAP kinase-2 is a component of a protein kinase cascade which becomes activated when cells are stimulated with interleukin-1 or tumour necrosis factor or exposed to cellular stresses (Rouse *et al.*, 1994; Cuenda *et al.*, 1995). MAPKAP kinase-2 phosphorylates PKB $\alpha$  stoichiometrically at Ser473 (Figure 7) and this finding was useful in establishing the role of Ser473 phosphorylation in regulating PKB $\alpha$  activity. However, although MAPKAP kinase-2 activity is stimulated to a small extent by insulin in L6 cells, no activation could be detected in 293 cells in response to insulin or IGF-1 (D.Alessi, unpublished experiments). Moreover, exposure of L6 cells or 293 cells to a chemical stress (0.5 mM sodium arsenite) strongly activated MAPKAP kinase-2 (D.Alessi, unpublished work), as found in other cells (Rouse *et al.*, 1994; Cuenda *et al.*, 1995), but did not activate PKB $\alpha$  at all. Furthermore, the drug SB 203580, which is a specific inhibitor of the protein kinase positioned immediately upstream of MAPKAP kinase-2 (Cuenda *et al.*, 1995), prevented activation of MAPKAP kinase-2 by arsenite but had no effect on activation of PKB $\alpha$  by insulin or IGF-1 (D.Alessi, unpublished experiments). Finally, activation of PKB $\alpha$  was prevented by wortmannin (Figures 2 and 5), but wortmannin had no effect on activation of MAPKAP kinase-2 by arsenite in L6 or 293 cells (D.Alessi, unpublished work). It should also be noted that the sequence surrounding Ser473 of PKB $\alpha$  (HFPQFSY) does not conform to the optimal consensus for phosphorylation by MAPKAP kinase-2, which requires Arg at position  $n - 3$  and a bulky hydrophobic residue at position  $n - 5$ , (where  $n$  is the position of the phosphorylated residue) (Stokoe *et al.*, 1993). The  $K_m$  for phosphorylation of the synthetic peptide comprising residues 465–478 is nearly 100-fold higher than the  $K_m$  for the standard MAPKAP kinase-2 substrate peptide (data not shown). It is therefore unlikely



that MAPKAP kinase-2 mediates phosphorylation of Ser473 *in vivo*.

The enzyme(s) which phosphorylates Thr308 and Ser473 *in vivo* does not appear to be PKB $\alpha$  itself. Thus incubation of the partially active Asp308 mutant with MgATP did not result in phosphorylation of Ser473, phosphorylation of the latter residue only occurring when MAPKAP kinase-2 was added (Figures 7A and 8). Similarly, Thr308 did not become phosphorylated when either the partially active Asp473 mutant or the partially active Ser473 phosphorylated form of PKB $\alpha$  were incubated with MgATP. PKB $\alpha$  when bound to lipid vesicles containing phosphatidylserine and PIP<sub>3</sub> also fails to activate upon incubation with MgATP (James *et al.*, 1996) and, after transfection into 293 cells, a 'kinase dead' mutant of PKB $\alpha$  became phosphorylated on Thr308 and Ser473 in response to insulin or IGF-1 (Figure 11). Furthermore, HA-PKB $\alpha$  from either unstimulated or insulin-stimulated 293 cells failed to phosphorylate the synthetic C-terminal peptide comprising amino acids 467–480 (M.Andjelkovic, unpublished work).

In unstimulated L6 myotubes, the endogenous PKB $\alpha$  was phosphorylated to a low level at a number of sites (Figure 2A), but in unstimulated 293 cells the transfected enzyme was heavily phosphorylated at Ser124 and Thr450 (Figure 6). Ser124 and Thr450 are both followed by proline residues, suggesting the involvement of 'proline-directed' protein kinases. Although, phosphorylation of Ser124 was greatly decreased when 'kinase dead' PKB $\alpha$  was transfected into 293 cells (Figure 10), it would be surprising if Ser124 is phosphorylated by PKB $\alpha$  itself, because the presence of a C-terminal proline abolishes the phosphorylation of synthetic peptides by PKB $\alpha$  (D.Alessi, unpublished work). Since transfected PKB $\alpha$  is inactive in unstimulated 293 cells (Figure 4A), phosphorylation of Ser124 and Thr450 clearly does not activate PKB $\alpha$  directly and further work is needed to understand the physiological roles of these modifications. Ser124 is located in the linker region between the PH domain and the catalytic domain of the mammalian PKB $\alpha$  isoform but, unlike Thr450, is not conserved in the *Drosophila* homologue (Andjelkovic *et al.*, 1995).

In summary, the present work and earlier studies suggest that agonists which activate PI 3-kinase are likely to stimulate PKB $\alpha$  activity via one of the following mechanisms. First, PIP<sub>3</sub> may activate one or more protein kinases which then phosphorylate PKB $\alpha$  at Thr308 and Ser473. Second, the formation of PIP<sub>3</sub> may lead to the recruitment of PKB $\alpha$  to the plasma membrane, where it is activated by a membrane-associated protein kinase(s). The membrane-associated Thr308 and Ser473 kinases might themselves be activated by PIP<sub>3</sub> and the possibility that Thr308 and/or Ser473 are phosphorylated directly by PI 3-kinase has also not been excluded, because this enzyme is known to phosphorylate itself (Dhand *et al.*, 1994) and other proteins (Lam *et al.*, 1994) on serine residues.

## Materials and methods

Tissue culture reagents, microcystin-LR and IGF-1 were obtained from Life Technologies Inc. (Paisley, UK), insulin from Novo-Nordisk (Bagsvaerd, Denmark), phosphate-free Dulbecco's minimal essential medium (DMEM) from ICN (Oxon, UK), protein G-Sepharose and

CH-Sepharose from Pharmacia (Milton Keynes, UK), alkylated trypsin from Promega (Southampton, UK) and 4-vinylpyridine, wortmannin and fluoroisothiocyanate-labelled anti-mouse IgG from goat from Sigma-Aldrich (Poole, UK). Polyclonal antibodies were raised in sheep against the peptides RPHFPQFSYASGTA (corresponding to the last 15 residues of rodent PKB $\alpha$ ) and MTSALATMRVDYEIQK (corresponding to residues 352–367 of human MAPKAP kinase-2) and affinity purified on peptide CH-Sepharose columns. Monoclonal HA antibodies were purified from the tissue culture medium of 12CA5 hybridoma and purified by chromatography on protein G-Sepharose. The peptide RPRHFPQFSYAS, corresponding to residues 465–478 of PKB $\alpha$ , was synthesized on an Applied Biosystems 430A peptide synthesizer. cDNA encoding residues 46–400 of human MAPKAP kinase-2 was expressed in *Escherichia coli* as a glutathione S-transferase fusion protein and activated with p38/RK by Mr A.Clifton (University of Dundee) as described previously (Ben-Levy *et al.*, 1995).

### Construction of expression vectors

The pECE constructs encoding the human HA-PKB $\alpha$  and 'kinase dead' (K179A) HA-KD-PKB $\alpha$  have already been described (Andjelkovic *et al.*, 1996). The mutants at Ser473 (HA-473A PKB $\alpha$  and HA-473D PKB $\alpha$ ) were created by PCR using a 5' oligonucleotide encoding amino acids 406–414 and mutating a 3' oligonucleotide encoding amino acids 468–480. The resulting PCR products were subcloned as a *CeIII*–*EcoRI* fragment into pECE.HA-PKB $\alpha$ . The Thr308 mutants (HA-308A PKB $\alpha$  and HA-308D PKB $\alpha$ ) were created by the two-stage PCR technique (No *et al.*, 1989) and subcloned as *NotI*–*EcoRI* fragments into pECE.HA-PKB $\alpha$ . The double mutant HA-308D/473D PKB $\alpha$  was made by subcloning the *CeIII*–*EcoRI* fragment encoding 473D into pECE.HA-308D PKB $\alpha$ . For construction of cytomegalovirus-driven expression constructs, *BglII*–*XbaI* fragments from the appropriate pECE constructs were subcloned into the same restriction sites of the pCMV5 vector (Andersson *et al.*, 1989). All constructs were confirmed by restriction analysis and sequencing and purified using Quiagen Plasmid Maxi or Mega Kits according to the manufacturer's protocol. All oligonucleotide sequences are available upon request.

### <sup>32</sup>P-labelling of L6 myotubes and immunoprecipitation of PKB $\alpha$

L6 cells were differentiated into myotubes on 10 cm diameter dishes (Hundal *et al.*, 1992). The myotubes were deprived of serum overnight in DMEM, washed three times in phosphate-free DMEM and incubated for a further 1 h with 5 ml medium. The myotubes were then washed twice with phosphate-free DMEM and incubated for 4 h with carrier-free [<sup>32</sup>P]orthophosphate (1 mCi/ml). Following incubation in the presence or absence of 100 nM wortmannin for 10 min, the myotubes were stimulated for 5 min at 37°C in the presence or absence of 100 nM insulin and placed on ice. The medium was aspirated, the myotubes washed twice with ice-cold DMEM buffer and then lysed with 1 ml ice-cold buffer A (50 mM Tris–HCl, pH 7.5, 1 mM EDTA 1 mM EGTA, 1% v/v Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1  $\mu$ M microcystin-LR, 0.27 M sucrose, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin and 0.1% v/v 2-mercaptoethanol). The lysates were centrifuged at 4°C for 10 min at 13 000 g and the supernatants incubated for 30 min on a shaking platform with 50  $\mu$ l protein G-Sepharose coupled to 50  $\mu$ g preimmune sheep IgG. The suspensions were centrifuged for 2 min at 13 000 g and the supernatants incubated for 60 min with 30  $\mu$ l protein G-Sepharose covalently coupled to 60  $\mu$ g PKB $\alpha$  antibody (Harlow and Lane, 1988). The protein G-Sepharose–antibody–PKB $\alpha$  complex was washed eight times with 1 ml buffer A containing 0.5 M NaCl and twice with 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA and 0.1% v/v 2-mercaptoethanol (buffer B).

### Assay of immunoprecipitated PKB $\alpha$ and protein determinations

Three aliquots of each immunoprecipitate (each comprising only 5% of the total immunoprecipitated PKB $\alpha$ ) were assayed for PKB $\alpha$  activity towards the peptide GRPRTSSFAEG as described previously (Cross *et al.*, 1995). One unit of activity was that amount which catalysed phosphorylation of 1 nmol substrate in 1 min. Protein concentrations were determined by the method of Bradford (1976).

### Tryptic digestion of *in vivo* phosphorylated PKB $\alpha$

The immunoprecipitated PKB $\alpha$  was added to an equal volume of 2% w/v SDS and 2% v/v 2-mercaptoethanol and incubated for 5 min at 100°C. After cooling to room temperature, 4-vinylpyridine was added

to a final concentration of 2% v/v and the mixture incubated for 1 h at 30°C on a shaking platform, followed by electrophoresis on a 10% polyacrylamide gel. After autoradiography, the 60 kDa band corresponding to rat PKB $\alpha$  was excised and the gel piece homogenized in 5 vol. 25 mM *N*-ethylmorpholine HCl, pH 7.7, containing 0.1% w/v SDS and 5% v/v 2-mercaptoethanol. The suspension was incubated for 1 h at 37°C on a shaking platform, then centrifuged for 1 min at 13 000 g and the supernatant collected. The pellet was incubated for a further 1 h with 5 vol. of the same buffer and centrifuged for 1 min at 13 000 g. The two supernatants (containing 80–90% of the  $^{32}$ P radioactivity) were combined, 0.2 vol. 100% w/v trichloroacetic acid added and the sample incubated for 1 h on ice. The suspension was centrifuged for 10 min at 13 000 g, the supernatant discarded and the pellet washed five times with 0.2 ml water. The pellet was then incubated at 30°C with 0.3 ml 50 mM Tris-HCl, pH 8.0, 0.1% v/v Triton X-100 containing 1  $\mu$ g alkylated trypsin. After 3 h another 1  $\mu$ g trypsin was added and the suspension left for a further 12 h. Guanidinium hydrochloride (8 M) was added to bring the final concentration to 1 M in order to precipitate any residual SDS and, after standing on ice for 10 min, the suspension was centrifuged for 5 min at 13 000 g. The supernatant containing 90% of the  $^{32}$ P radioactivity was chromatographed on a Vydac C18 column as described in the legend to Figure 2.

#### **Transfection of 293 cells and immunoprecipitation of HA-tagged PKB $\alpha$**

Human embryonic kidney 293 cells were cultured at 37°C in an atmosphere of 5% CO $_2$  on 10 cm diameter dishes in DMEM containing 10% fetal calf serum. Cells were split to a density of  $2 \times 10^6$  per 10 cm dish and after 24 h at 37°C the medium was aspirated and 10 ml freshly prepared DMEM containing 10% fetal calf serum added. Cells were transfected by a modified calcium phosphate method (Chen and Okayama, 1988) with 1  $\mu$ g/ml DNA/plasmid. Aliquots of 10  $\mu$ g plasmid DNA in 0.45 ml sterile water were added to 50  $\mu$ l sterile 2.5 M CaCl $_2$  and then 0.5 ml sterile buffer composed of 50 mM *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid-HCl, pH 6.96, 0.28 M NaCl and 1.5 mM Na $_2$ HPO $_4$  was added. The resulting mixture was vortexed for 1 min, allowed to stand at room temperature for 20 min and then added dropwise to a 10 cm dish of 293 cells. The cells were placed in an atmosphere of 3% CO $_2$  for 16 h at 37°C, then the medium was aspirated and replaced with fresh DMEM containing 10% fetal calf serum. The cells were incubated for 12 h at 37°C in an atmosphere of 5% CO $_2$  and then for 12 h in DMEM in the absence of serum. Cells were preincubated for 10 min in the presence of 0.1% DMSO or 100 nM wortmannin in 0.1% DMSO and then stimulated for 10 min with either 100 nM insulin or 50 ng/ml IGF-1 in the continued presence or absence of wortmannin. After washing twice with ice-cold DMEM the cells were lysed in 1 ml ice-cold buffer A, the lysate was centrifuged at 4°C for 10 min at 13 000 g and an aliquot of the supernatant (10  $\mu$ g protein) was incubated for 60 min on a shaking platform with 5  $\mu$ l protein G-Sepharose coupled to 2  $\mu$ g HA monoclonal antibody. The suspension was centrifuged for 1 min at 13 000 g, the protein G-Sepharose-antibody-HA-PKB $\alpha$  complex washed twice with 1 ml buffer A containing 0.5 M NaCl and twice with buffer B and the immunoprecipitate assayed for PKB $\alpha$  activity as described above.

#### **$^{32}$ P-Labeling of 293 cells transfected with HA-PKB $\alpha$**

293 cells transfected with HA-PKB $\alpha$  DNA constructs were washed with phosphate-free DMEM, incubated with [ $^{32}$ P]orthophosphate (1 mCi/ml) as described for L6 myotubes, then stimulated with insulin or IGF-1 and lysed and PKB $\alpha$  immunoprecipitated as described above. The  $^{32}$ P-labelled HA-PKB $\alpha$  immunoprecipitates were washed, alkylated with 4-vinylpyridine, electrophoresed and digested with trypsin as described above for the endogenous PKB $\alpha$  present in rat L6 myotubes.

#### **Transfection of COS-1 cells and immunoprecipitation of HA-PKB $\alpha$**

COS-1 cells were maintained in DMEM supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO $_2$ . Cells at 70–80% confluency were transfected by a DEAE-dextran method (Seed and Aruffo, 1987) and 48 h later serum starved for 24 h. Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Nonidet P-40, 25 mM NaF, 40 mM sodium  $\beta$ -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM EDTA, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (buffer C) and lysates centrifuged for 15 min at 13 000 g at 4°C. Supernatants were precleared once for 30 min at 4°C with 0.1 vol. 50% Sepharose 4B/25% Pansorbin (Pharmacia and Calbiochem respectively) and HA-PKB $\alpha$  immunoprecipitated from 1

mg extract using the 12CA5 antibody coupled to protein A-Sepharose beads. Immunoprecipitates were washed twice with buffer C containing 0.5 M NaCl and once with buffer C.

#### **Immunoblotting and quantification of levels of PKB $\alpha$ expression**

Cells extracts were resolved by 10% SDS-PAGE and transferred to Immobilon membranes (Millipore). Filters were blocked for 30 min in a blocking buffer containing 5% skimmed milk in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100 and 0.5% Tween 20, followed by a 2 h incubation with the 12CA5 supernatant 1000-fold diluted in the same buffer. The secondary antibody was alkaline-conjugated anti-mouse Ig from goat (Southern Biotechnology Associates Inc) 1000-fold diluted in the blocking buffer. Detection was performed using AP colour development reagents from BioRad according to the manufacturer's instructions. Quantification of levels of PKB $\alpha$  expression was achieved by chemiluminescence, using fluroisothiocyanate-labelled anti-mouse IgG from goat as the secondary antibody and the Storm 840/860 and ImageQuant software from Molecular Dynamics.

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